

# Multiple infection of porcine Torque teno virus in a single pig and characterization of the full-length genomic sequences of four U.S. prototype PTTV strains: Implication for genotyping of PTTV

Y.W. Huang, Y.Y. Ni, B.A. Dryman, X.J. Meng\*

Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, 1981 Kraft Drive, Blacksburg, VA 24061-0913, USA

## ARTICLE INFO

### Article history:

Received 26 August 2009  
Returned to author for revision  
23 September 2009  
Accepted 19 October 2009  
Available online 13 November 2009

### Keywords:

Torque teno virus (TTV)  
Porcine TTV (PTTV)  
Anellovirus  
Multiple infection  
Pig  
Genomic sequences  
Genotyping

## ABSTRACT

Porcine Torque teno virus (PTTV) was recently shown to partially contribute to the experimental induction of porcine dermatitis and nephropathy syndrome and postweaning multisystemic wasting syndrome in pigs in the United States. We report here the identification of four distinct full-length genomic sequences of PTTV strains from a single pig in Virginia. Detailed analyses of the genomic organization, the degree of variability and the characteristics of conserved nucleotide and amino acid motifs of PTTV were conducted. The results showed that these four prototype U.S. strains of PTTV identified from the same pig represent distinct genotypes or subtypes and a revised classification system for PPTV is subsequently proposed. This is the first study documenting multiple PTTV infections with distinct genotypes or subtypes in a single pig. The identification of novel PTTV strains from pigs in the United States also pave the way for future disease characterization and genotyping of PTTV.

© 2009 Elsevier Inc. All rights reserved.

## Introduction

Torque teno virus (TTV) was first discovered in a Japanese patient with post-transfusion non-A-E hepatitis in 1997 (Nishizawa et al., 1997). Since then, a large number of human TTV strains and two groups of TTV-related viruses, designated subsequently as Torque teno mini virus (TTMV) and Torque teno midi virus (TTMDV), have been identified with high prevalence in serum and other tissues from healthy humans (Hino and Miyata, 2007; Okamoto, 2009a). Human TTV, TTMV and TTMDV are non-enveloped spherical viruses with circular single-stranded DNA (ssDNA) genomes of 3.6–3.9, 2.8–2.9 and 3.2 kb in length, respectively, and they are currently classified into a floating genus *Anellovirus* by the International Committee on Taxonomy of Viruses (ICTV) (Biagini, 2009). These three groups of TTV-related viruses exhibit a high degree of genetic heterogeneity, each consisting of many genogroups and genotypes (Biagini et al., 2006; Jelcic et al., 2004). The prevalence of multiple infection of TTV with different genotypes as well as dual or triple infections of TTV, TTMV and TTMDV have been documented in humans and are considered to

be a common event in healthy human adults (Niel et al., 2000; Ninomiya et al., 2008; Okamoto, 2009a; Takayama et al., 1999).

TTV infects not only humans but various other animal species as well including non-human primates, tupaia, pigs, cattle, cats, dogs and sea lions (Biagini et al., 2007; Inami et al., 2000; Ng et al., 2009; Okamoto, 2009b; Okamoto et al., 2001; Okamoto et al., 2000a; Okamoto et al., 2002). In addition, chimpanzees are also infected with TTMV and TTMDV (Ninomiya et al., 2009; Okamoto et al., 2000a). Although the genomic sizes of the identified animal TTV strains, especially non-primate animal TTV, are relatively smaller than that of human TTV, they share the same genomic structure with a minimum of two partially overlapping open reading frames (ORF1 and ORF2) translated from the negative ssDNA as well as a short stretch of untranslated region (UTR) with high GC content (~90%) (Okamoto, 2009b). The arrangement of TTV ORFs is quite similar to that of chicken anemia virus (CAV) belonging to the genus *Gyrovirus* in the family *Circoviridae* but is different from porcine circovirus (PCV) types 1 (PCV1) and 2 (PCV2), which are also classified into the same family (Davidson and Shulman, 2008; Hino and Prasetyo, 2009). The genomes of PCV1 and PCV2 are ambisense, in which the ORF1 is coded for by the genomic strand and the ORF2 is coded for by the antigenomic strand (Hino and Miyata, 2007). Recently, the transcription pattern and translated products of both human TTV genotypes 1 and 6 have been identified by transfection of the respective TTV infectious DNA clones into cultured cells (Mueller et al., 2008; Qiu

Abbreviations: TTV, Torque teno virus; PTTV, porcine Torque teno virus; TTMV, Torque teno mini virus; TTMDV, Torque teno midi virus; PCV, porcine circovirus; CAV, chicken anemia virus; UTR, untranslated region.

\* Corresponding author. Fax: +1 540 231 3414.

E-mail address: [xjmeng@vt.edu](mailto:xjmeng@vt.edu) (X.J. Meng).

et al., 2005). Expression of at least six proteins, designated ORF1, ORF2, ORF1/1, ORF2/2, ORF1/2 and ORF2/3, from three or more spliced mRNAs, have been reported (Kakkola et al., 2009; Mueller et al., 2008; Qiu et al., 2005). Accordingly, it is likely that, when more data regarding the animal TTV become available, the presumed genome structure of animal TTV will need to be modified.

Although TTV was first identified in a cryptogenic hepatitis patient, subsequent studies were not able to produce evidence of a significant role of TTV in the pathogenesis of hepatitis or other diseases (Hino and Miyata, 2007; Maggi and Bendinelli, 2009; Okamoto, 2009a). While human TTV is not considered to be directly associated with a disease, porcine TTV (PTTV) was recently shown to partially contribute to the experimental induction of porcine dermatitis and nephropathy syndrome (PDNS) combined with porcine reproductive and respiratory syndrome virus (PRRSV) infection (Krakowka et al., 2008), and also to the experimental induction of postweaning multisystemic wasting syndrome (PMWS) combined with PCV2 infection in a gnotobiotic pig model (Ellis et al., 2008). The data suggested that porcine TTV is pathogenic in pigs. However, further in-depth studies with a biologically pure form of PTTV virus to definitively characterize the diseases and lesions associated with PTTV infection are needed.

Compared to human TTV, the genomic information of PTTV is very limited. Currently, only one full-length and two near full-length genomic sequences of PTTV are reported from pigs in Japan and Brazil, respectively (Niel et al., 2005; Okamoto et al., 2002). Among the three known PTTV strains, the Sd-TTV31 and TTV-1p strains were clustered together into the genogroup 1 (PTTV1), whereas TTV-2p was the sole strain classified into the genogroup 2 (PTTV2) (Niel et al., 2005). However, genogroup classification is a vague concept in the taxonomy of virology, and further and more accurate classification of PTTV is needed but can only be performed when more full-length genomic sequences of new PTTV strains representing multiple genotypes become available.

We previously showed that PTTV infections were widespread in pigs from six different countries including the United States, Canada, Spain, China, Korea and Thailand (McKeown et al., 2004). In the present study, we report the identification of four distinct full-length genomic sequences of PTTV strains representing different genotypes or subtypes in a serum sample of a single boar in Virginia, USA. The results from this study provided, for the first time, a definite evidence for multiple PTTV infections with distinct genotypes or subtypes in a single pig.

## Results and discussion

### *Screening for porcine TTV-positive samples collected from boars in a farm from Virginia*

We previously detected porcine TTV DNA from pigs in different geographic regions by nested PCR based on the UTR sequence of a Japanese PTTV1 strain Sd-TTV31 (McKeown et al., 2004). With the recent identification of PTTV2, two different sets of nested PCR primers have been used to amplify region A of PTTV1 and region D of PTTV2, respectively (Fig. 1A) (Ellis et al., 2008; Kekarainen et al., 2006; Krakowka et al., 2008). A similar detection approach was also utilized in the present study to identify PTTV strains from pigs in the United States. In order to screen for indigenous PTTV1- or PTTV2-positive samples for subsequent use to determine the full-length genomic sequences, 20 sera (SR#1–20) and 19 semen samples (SM#1–18 and SM#20) collected from 20 boars in a farm of Virginia were subjected to nested PCR analyses. Surprisingly, all the 20 serum samples were positive for PTTV1 and 19 were also positive for PTTV2 (except for SR#18). In contrast, only one semen sample (SM#6) was PTTV1-positive and three semen samples (SM#8, 9 and 20) were PTTV2-positive. The result was consistent with a recent study in that boar semen samples were shown to be positive for PTTV DNA in Spain

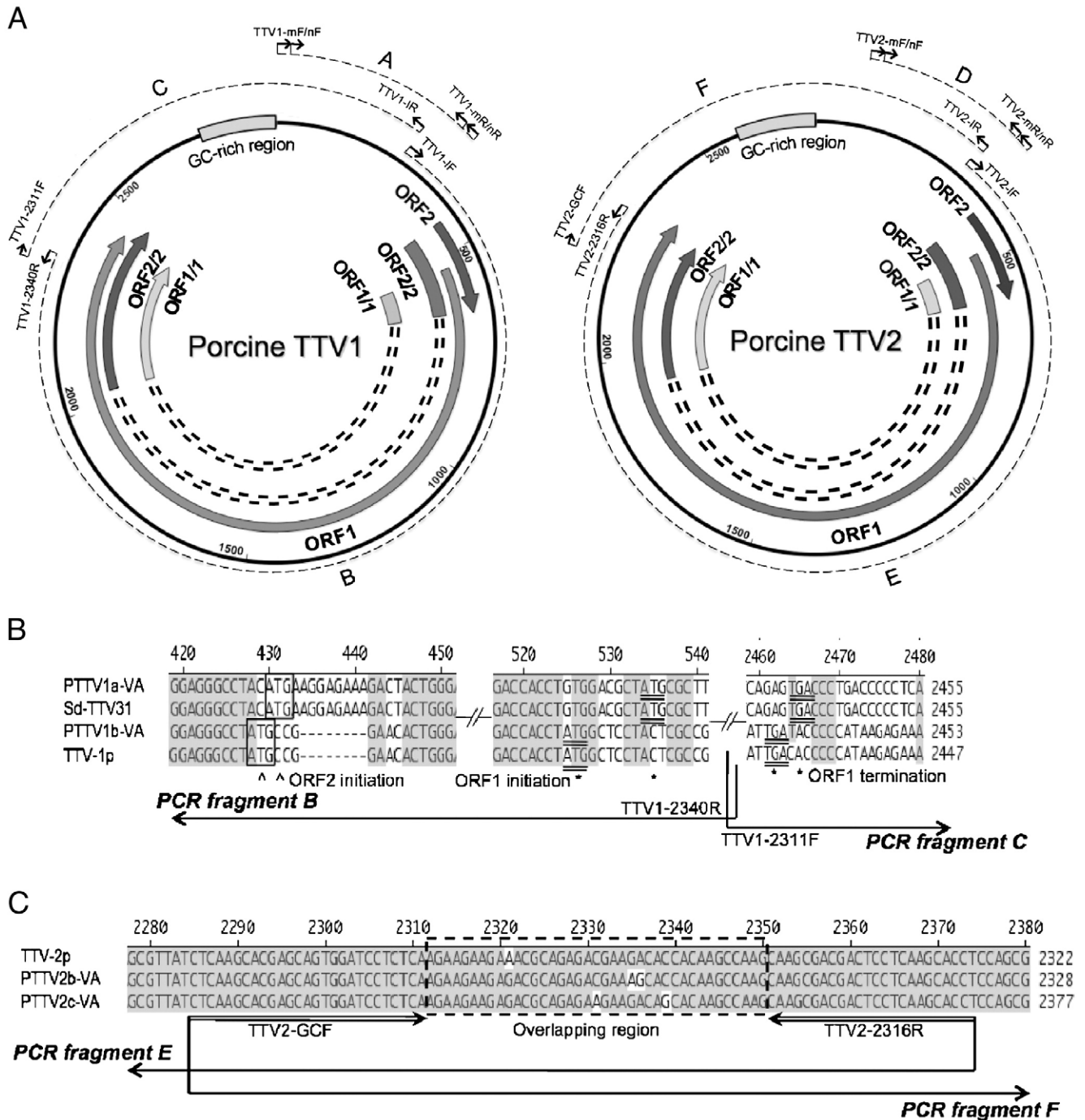
(Kekarainen et al., 2007) and thus suggesting a potential vertical transmission of PTTV. However, the prevalence rates of both PTTV1 and PTTV2 in semen were much lower than that in sera, suggesting that there is no direct association for the presence of PTTV DNAs in sera and semen of the same pig.

### *Identification and cloning of four distinct porcine TTV strains from a serum sample of a single boar in the United States*

One boar serum sample (SR#5) that was shown to be positive for both PTTV1 and PTTV2 in the first-round PCR, thus indicative of higher virus load, was used for subsequent full-length genomic cloning of PTTV. We initially attempted to utilize two primer sets (NG372/NG373 and NG384/NG385) of an inverse PCR (Okamoto et al., 2002) designed for cloning of the first PTTV strain Sd-TTV31 to amplify the virus genomic DNA. However, no PCR product was obtained after several trials. Based upon the initial sequence of the region A of PTTV1 and the region D of PTTV2, we subsequently designed two new pairs of primers (TTV1-IF/TTV1-2340R and TTV1-2311F/TTV1-TTV-IR) to amplify regions B and C spanning the assumed PTTV1 genome and two additional pairs of primers (TTV2-IF/TTV2-2316R and TTV2-GCF/TTV2-TTV-IR) to amplify regions E and F spanning the assumed PTTV2 genome, respectively (Fig. 1A and Table 1). Primers TTV1-2340R and TTV1-2311F were deduced from a common sequence in PTTV1 strains Sd-TTV31 (Okamoto et al., 2002) and TTV-1p (Niel et al., 2005) that is absent in PTTV2 strain TTV-2p (Niel et al., 2005), whereas primers TTV2-2316R and TTV2-GCF were deduced from a sequence of strain TTV-2p that is absent in the two PTTV1 strains. The resulting four different PCR products with expected sizes were each inserted into a blunt-end cloning vector, and the resulting recombinant plasmids were transformed into *Escherichia coli*. Eight to fifteen positive (with white color) bacterial clones for each construct representing fragments B, C, E and F were identified and subsequently sequenced.

Unexpectedly, two groups of sequence data from each construct were identified, indicating that there exist two types of PTTVs in genogroup 1 and genogroup 2 from the same pig. In order to differentiate and assemble the four PTTV strains, sequence comparisons were performed together with the three known PTTV strains, Sd-TTV31, TTV-1p and TTV-2p (Figs. 1B and C). For PTTV1, the initiation codon ATG and the termination codon TGA of the putative ORF1 were located in fragments B and C, respectively (Fig. 1B). The positions of the codons were differed in two PTTV1 groups, the first one identical to Sd-TTV31 and the second one identical to TTV-1p (Fig. 1B). In addition, the ORF2 initiation codons in the two groups were also located at different positions consistent with that of ORF1. Moreover, phylogenetic analyses using four different sequences of the region B (two from the sequencing data and two from strains Sd-TTV31 and TTV-1p) and four different sequences of the region C supported that the first sequence was clustered with Sd-TTV31 and the second was clustered with TTV-1p (data not shown). Therefore, we were able to differentiate and assemble two groups of sequence data from both fragments B and C into two full-length PTTV1 genomes that were designated as strains PTTV1a-VA and PTTV1b-VA, respectively (Fig. 1B).

Differentiation of the two PTTV2 strains was easier. A unique continuous “AG” nucleotides located in the overlapping region of two PCR fragments was shared by two groups of sequence data from fragments E and F, respectively (Fig. 1C). The assembled full-length genomic sequence represented a PTTV2 strain and was designated as PTTV2b-VA. Similarly, the complete genomic sequence of a second strain designated as PTTV2c-VA was assembled based upon two unique single “A” and “G” nucleotides shared in the overlapping region by another set of sequence data from fragments E and F, respectively (Fig. 1C). Phylogenetic analyses using four sequences from fragments E and F together with the two corresponding sequences from TTV-2p also supported this assignment (data not shown).



**Fig. 1.** Schematic diagram of genomic structures, strategies for genomic cloning and assemblies of four prototype U.S. strains of porcine TT virus group 1 (species 1) and group 2 (species 2) strains. (A) Both the PTTV1 and PTTV2 genomes are shown in bold and the sizes and directions of the four putative ORFs (ORF1, ORF2, ORF1/1 and ORF2/2) are indicated by arrows. The GC-rich regions are also shown. Dashed-line arcs A and D represent the regions used for detection of PTTV1 and PTTV2 from serum and semen samples by nested PCR, respectively. Dashed-line arcs B and C represent the two overlapping PCR fragments for genomic cloning of PTTV1 whereas dashed-line arcs E and F represent the two overlapping PCR fragments for genomic cloning of PTTV2. The locations of the primers used in the study (see Table 1) are also shown in the corresponding positions. (B) Differentiation and assembly of full-length genomic sequences of PTTV1 strains PTTV1a-VA and PTTV1b-VA with PCR fragments B and C that were subsequently cloned. The initiation codons of ORF1 and ORF2 in the fragment B as well as the termination codons of ORF1 in the fragment C are marked by “^” or “\*”. The corresponding sequences of two known PTTV1 strains, Sd-TTV31 and TTV-1p, are also shown. Conserved sequences are shaded, and dashes indicate nucleotide deletions. (C) Differentiation and assembly of full-length genomic sequences of PTTV2 strains PTTV2b-VA and PTTV2c-VA with PCR fragments E and F that were subsequently cloned. The corresponding sequence of TTV-2p strain is included and the conserved sequences are shaded. Dashes indicate nucleotide deletions. The unique nucleotides within the overlapping region (boxed with dashed-line) for each strain (a continuous “AG” nucleotides for PTTV2b-VA and two single “A” and “G” nucleotides for PTTV2c-VA) are shown, respectively.

#### Proposed genomic structure of the four prototype U.S. strains of porcine TTV identified from a single pig

The proposed genomic structure for each of the four PTTV strains was analyzed together with the three known PTTV strains, Sd-TTV31, TTV-1p and TTV-2p. All the four U.S. strains of PTTV have a

similar genomic size of 2,878 bp (PTTV1a-VA), 2,875 bp (PTTV1b-VA), 2,750 bp (PTTV2b-VA), and 2,803 bp (PTTV2c-VA), respectively. The published sequences of the strains TTV-1p and TTV-2p all have undetermined nucleotides in the GC-rich region of the UTR. After artificial filling of these nucleotides with the consensus sequences corresponding to PTTV1 and PTTV2, it was shown that



**Table 1**

Oligonucleotide primers used for nested PCR and genomic PCR amplifications of porcine TT viruses.

Primer ID	Sequence (5' to 3')	Used for:
TTV1-mF	TACACTTCCGGTTCAGGAGGCT	Detection of porcine TTV1
TTV1-mR	ACTCAGCCATTCGGAACCTCAC	Detection of porcine TTV1
TTV1-nF	CAATTGGCTCGTTCGCTCGC	Detection of porcine TTV1
TTV1-nR	TACTTATATTCGTTTCGTGGGAAC	Detection of porcine TTV1
TTV2-mF	AGTTACACATAACCACCAAAACC	Detection of porcine TTV2
TTV2-mR	ATTACCGCTGCCGATAGGC	Detection of porcine TTV2
TTV2-nF	CCAAACCACAGAAACTGTGC	Detection of porcine TTV2
TTV2-nR	CTTGACTCCGCTCTCAGGAG	Detection of porcine TTV2
TTV1-IF	CATAGGGTGTAAACCAATCAGATTTAAGGCGTT	Genomic cloning (fragment B)
TTV1-2340R	GGTCATCAGACGATCCATCTCCCTCAG	Genomic cloning (fragment B)
TTV1-2311F	CTTCTGAGGGAGATGGATCGTCTGATGA	Genomic cloning (fragment C)
TTV1-IR	TTGAGCTCCCGACCAATCAGAATTGACT	Genomic cloning (fragment C)
TTV2-IF	TTGTGCCGGAGCTCTGAGAGC	Genomic cloning (fragment E)
TTV2-2316R	AGGTGCTTGAGGAGTCGTCGCTTG	Genomic cloning (fragment E)
TTV2-GCF	CTCAAGCACGAGCAGTGATCCTCTCA	Genomic cloning (fragment F)
TTV2-IR	TACCCAGGCGGTAGACACTCAGCTCT	Genomic cloning (fragment F)

the TTV-1p is more closely related to PTTV1b-VA, and that TTV-2p is more closely related to PTTV2b-VA in genomic length, respectively (data not shown).

Two recent studies have identified the transcribed viral mRNAs and the expression of at least six viral proteins during human TTV replication (Mueller et al., 2008; Qiu et al., 2005), which is more than the predicted number of ORFs encoded by human TTV (Okamoto et al., 2000b), therefore we included the new human TTV genomic information for comparison with the PTTV sequences. The 5'-ends of the mRNA transcripts of human TTV strain P/1C1 were mapped to an "A" that is 25 nt downstream of the TATA-box (Mueller et al., 2008). This starting point, its adjacent sequence (CGAATGGCTGAGTTATGCGGC; the starting point was underlined) and the distance to the upstream TATA-box (24 nt) are very conserved in all seven PTTV strains, suggesting that PTTV and human TTV may utilize a common 5'-end of mRNA for translation.

Five additional completely-conserved regions were identified in the vicinity of the TATA-box among all seven PTTV strains. Two regions of 11 nt each (AGTCCTCATTT and AACCAATCAGA) are located in the upstream of the TATA-box, whereas the remaining three regions (CTGGGCGG-GTGCCGGAG of 17 nt; CGGAGTCAAGGGGC of 14 nt; TATCGGGCAGG of 11nt) are located between the proposed 5'-end of mRNA and the initiation codon of ORF2. These conserved PTTV-specific sequences may contain the common elements regulating the viral gene expression.

Previously, three ORFs (ORFs 1–3) were proposed in the genome of the three known PTTV strains, respectively (Niel et al., 2005; Okamoto et al., 2002). The four prototype U.S. strains of PTTV identified in this study possess this structure. The corresponding ORF3 in human TTV has been renamed as ORF2/2 since it initiates at the same ATG in ORF2 and remains in the same ORF (extending ORF2) after the splicing (Fig. 1A) (Mueller et al., 2008; Qiu et al., 2005). We follow the nomenclature of human TTV for revising PTTV classification in this study. Human TTV ORF1/1 is a newly identified viral protein that is encoded by two exons in ORF1 (Qiu et al.,

2005). ORF1/1 share the identical N- and C-terminal part with ORF1. The PTTV ORF1/1 counterpart was readily identified in all seven PTTV strains (Fig. 1A).

The ORF1 and ORF2 are encoded by a ~2.8 kb viral mRNA whereas the ORF1/1 and ORF2/2 are encoded by a spliced viral mRNA with ~1.2 kb in human TTV (Mueller et al., 2008; Qiu et al., 2005). Since these four ORFs were also deduced in PTTV genomes and since the sequences and positions of the putative splice donor and acceptor sites in the seven PTTV strains are very conserved, we speculate that porcine TTV probably also encodes the two corresponding mRNAs. Whether more spliced mRNAs and their encoding proteins exist in PTTV, as shown in human TTV, remains to be determined in future studies.

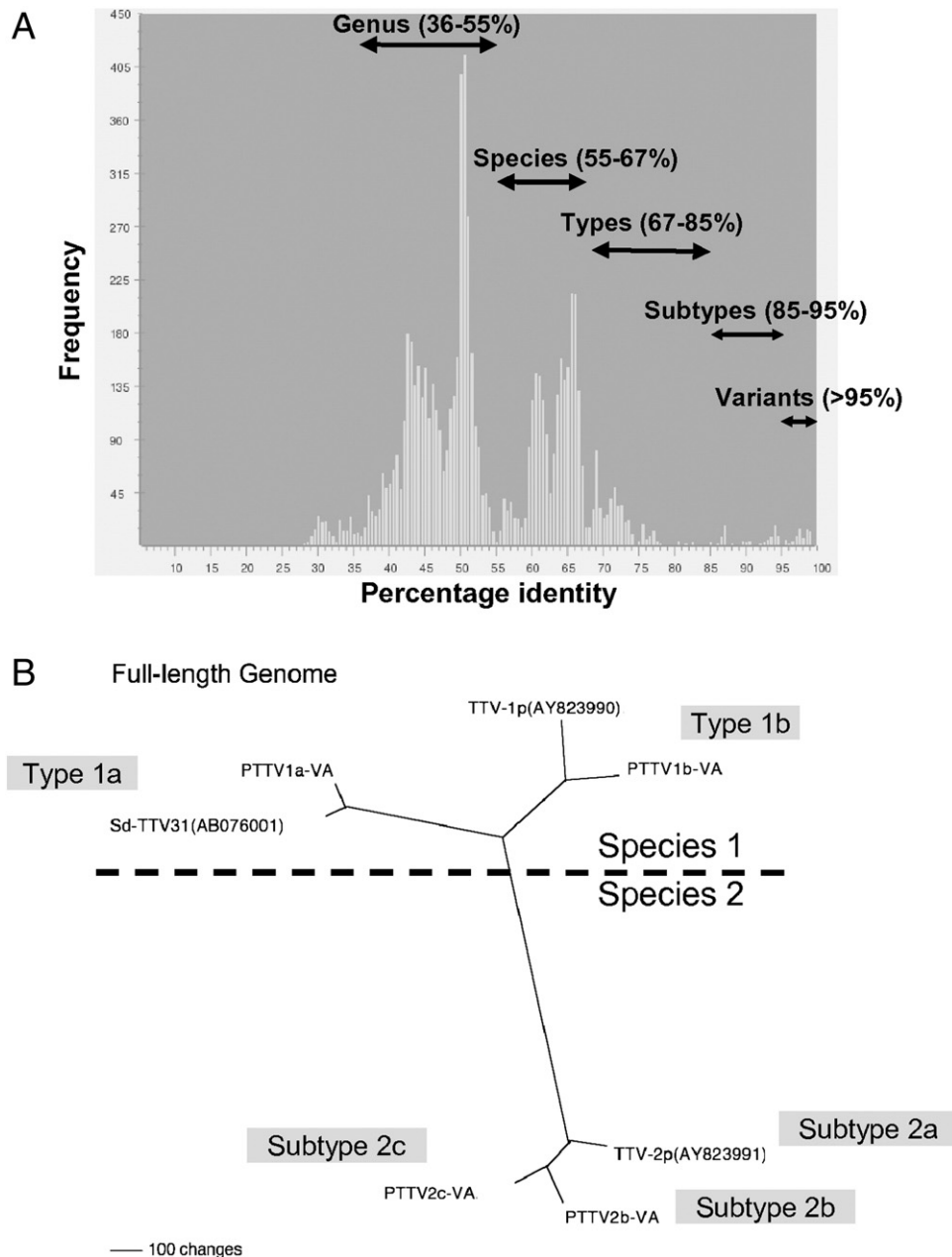
Most of the human TTV strains share a genetic similarity with the CAV, encoding a TTV apoptosis-inducing protein (TAIP) in which its CAV counterpart was named apoptin (de Smit and Noteborn, 2009). The ORF of TAIP is embedded within the ORF2. However, the corresponding TAIP does not exist in porcine TTV. A recent study showed that the expression of apoptin or TAIP was required for CAV replication in cultured cells (Prasetyo et al., 2009). It will be interesting to investigate the effects of an introduced TAIP on PTTV replication in the future.

#### Phylogenetic relationship of the seven known PTTV strains

Because of a high degree of genetic diversity among human TTV strains, a new family *Anelloviridae* may be established in the near future (Biagini, 2009). Currently, the TTV classification was mainly focused on the human TTV and its related TTMV and TTMDV. Other TTV strains identified from animal species are not adequately considered in the classification due to the limited number of available full-length genomic sequences. For porcine TTV, although sequencing data based on the partial UTR are continuously added to the GenBank database, the use of this conserved short UTR region (<8% of the full-length genome) for phylogenetic analyses is problematic and inaccurate.

Pairwise sequence comparisons (PASC) is a useful method that plots the frequency distribution of pairwise nucleotide sequence identity percentages from all available genomic sequence of viruses in the same family (Bao et al., 2008). The different peaks generated by the PASC program usually represent groups of virus genera, species, types, subtypes and strains (Fig. 2A). In this study, we performed PASC analyses of TTV using 121 full-length genomic sequences of human and animal TTV-related strains available in GenBank database (Fig. 2A). Assuming that TTV members are classified into a separate family, *Anelloviridae*, the two major peaks, at 36–55% and 55–67% nucleotide sequence identities, represent groups of genera and species, respectively (Fig. 2A). Accordingly, a TTV type is defined as a group of TTV having 67–85% nucleotide sequence identity whereas a TTV subtype may be defined as a group of TTV sequences sharing 85–95% nucleotide sequence identity. TTV strains sharing more than 95% nucleotide sequence identity may be further classified into variants (Fig. 2A). A similar classification has been proposed using sequences of 103 TTV isolates by Jelcic et al. (2004).

This proposed criteria of TTV classification were applied to phylogenetic analyses of the genomic sequences of the four prototype U.S. strains of PTTV and the three other known PTTV strains. Pairwise comparison of full-length nucleotide sequences among these strains showed that the four PTTV1 strains have 54.0–56.4% nucleotide sequence identity compared to the three PTTV2 strains (Table 2). Therefore, the previously designated "genogroup" of PTTV in the literature will probably be more appropriate to designate as "species", and PTTV1 and PTTV2 probably should represent porcine TTV species 1 and species 2, respectively. PTTV species 1 consists of two types of viruses designated as type 1a



**Fig. 2.** Phylogenetic analyses. (A) PASC (pairwise sequence comparisons) distribution of nucleotide sequence comparisons of 121 TTV strains available in GenBank database. The genus, species, types, subtypes and variants and their corresponding percentage of nucleotide sequence identities are displayed. (B) Phylogenetic tree constructed by the neighbor-joining method based upon the full-length genomic nucleotide sequences among seven porcine TTV strains. The newly-proposed PTTV species, types and subtypes are shown in the phylogenetic tree.

(including Sd-TTV31 and PTTV1a-VA) and type 1b (including TTV-1p and PTTV1b-VA), respectively, since the nucleotide sequence identity between these two types of viruses are between 69.8% and 70.7% (Table 2). Sd-TTV31 and TTV1a-VA are recognized as variant strains of the same species due to their higher sequence identity (95.1%). However, the two type 1b strains, TTV-1p and PTTV1b-VA, may belong to two different subtypes (nucleotide sequence identity = 86.4%). For PTTV species 2, three strains are likely to be classified into separate subtypes (TTV-2p for subtype 2a, PTTV2b-VA for subtype 2b, and PTTV2c-VA for subtype 2c, respectively) based upon their 86.5–90.9% nucleotide sequence identity. This proposed new classification system for PTTV was clearly evident in the phylogenetic tree (Fig. 2B). Phylogenetic trees constructed based upon the deduced amino acid sequences of ORF1, ORF1/1, ORF2 and

ORF2/2 of PTTV were also consistent with this proposed classification (data not shown). However, it should be pointed out that, due to the limited number of porcine TTV strains with available full-length genomic sequences, the proposed classification of PTTV here will be likely modified in the future when additional full-length PTTV sequences become available. The “subtype” designations for classification of PTTV2 strains in this study are tentative.

Unique mutations and deletions and/or insertions are scattered throughout the genomes between PTTV species, types and subtypes. For examples, the location of ORF1 initiation and termination codons and the ORF2 initiation codons between PTTV type 1a and 1b, which was shown in Fig. 1B as mentioned above, are different. The two PTTV1b strains also have a two-codon deletion after the ORF2 initiation compared to PTTV1a (Fig. 1B).

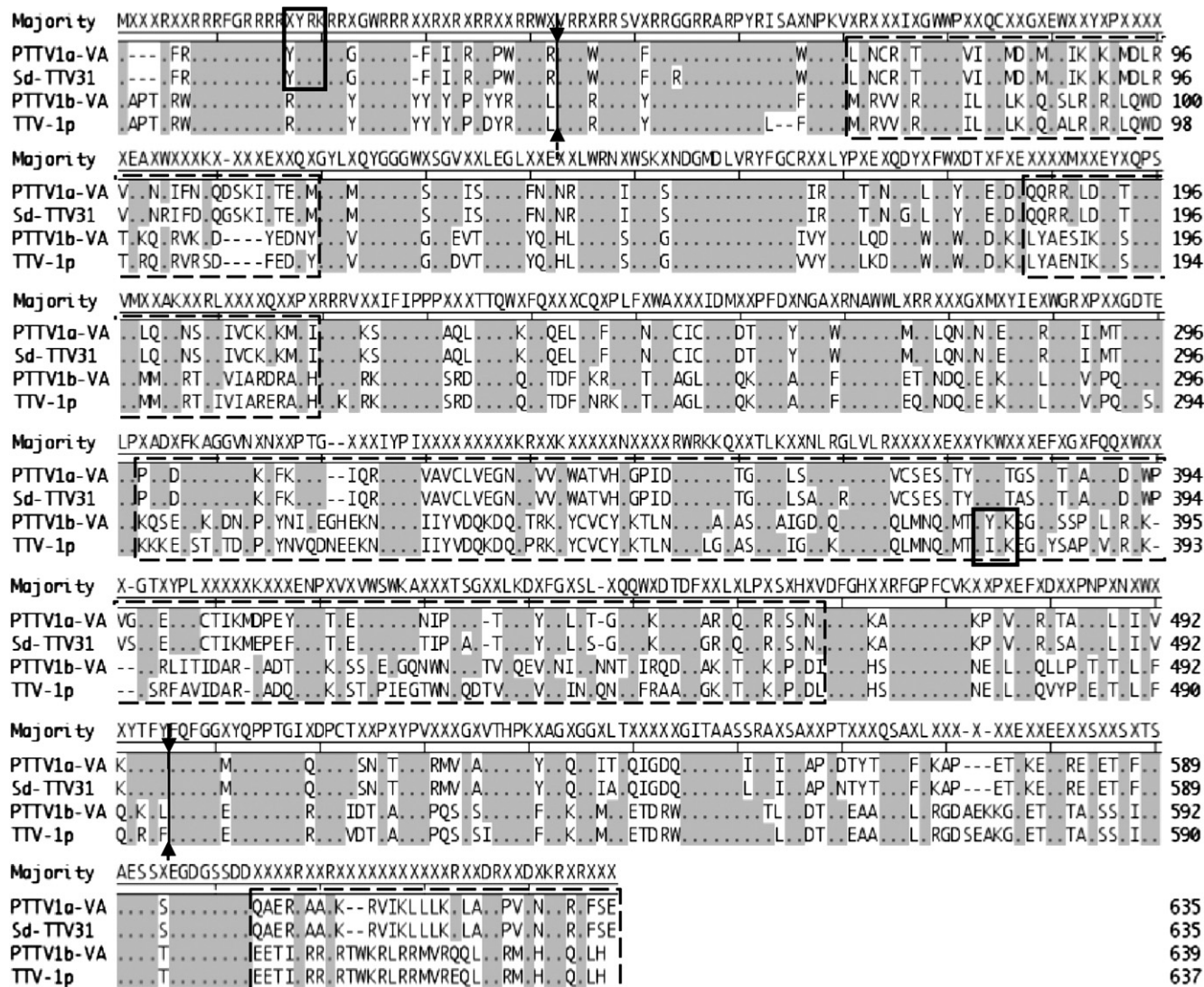
**Table 2**  
Pairwise sequence comparison of the full-length genomic sequence of the seven porcine TTV strains.

	Porcine TTV species 1				Porcine TTV species 2		
	Type 1a		Type 1b		Subtype 2a	Subtype 2b	Subtype 2c
	PTTV1a-VA	Sd-TTV31	PTTV1b-VA	TTV-1p	TTV-2p	PTTV2b-VA	PTTV2c-VA
Type 1a							
PTTV1a-VA	–	95.1	70.5	69.8	55.7	55.1	56.2
Sd-TTV31		–	70.7	70.1	55.9	56.0	56.4
Type 1b							
PTTV1b-VA			–	86.4	54.0	54.7	55.2
TTV-1p				–	55.2	54.7	55.4
Subtype 2a							
TTV-2p					–	86.5	86.8
Subtype 2b							
PTTV2b-VA						–	90.9
Subtype 2c							
PTTV2c-VA							–

The data were generated by using the PASC program, and the values indicate % nucleotide sequence identities.

Remarkably, both TTV-2p and PTTV2b-VA have a large 52-nt deletion, which is 39 nt upstream of the first 11-nt conserved sequence (AGTCTCATTT) in the UTR, compared to PTTV2c-VA. Due to

this deletion, the genomic size of PTTV2b-VA (probably TTV-2p as well) was significantly smaller than that of PTTV2c-VA. A number of "subviral" human TTV clones have been isolated from serum samples



**Fig. 3.** Alignment of the full-length amino acid sequences of ORF1 among four PTTV1 strains. Conserved sequences are indicated by dots and shaded. Dashes indicate amino acid deletions. The RCR motifs are boxed with solid lines. The four regions with a relatively high number of aa substitutions are boxed with dashed lines. The connection boundaries of ORF1/1 are indicated by arrows.



that are considered as full-length TTV genomes since the ORFs in a majority of these subviral molecules usually remain intact (de Villiers et al., 2009; Leppik et al., 2007). They have variable lengths in the UTR that are completely or partially deleted. The situation of TTV-2p and PTTV2b-VA appears to resemble that of the human TTV subviral molecules, implying that subtypes PTTV2a and PTTV2b might be the subviral molecules derived from subtype PTTV2c. Of note, the 3'-terminal sequence of a nested PCR primer TTV2-nF (Table 1) that is commonly used for detection of the PTTV2 from field samples by other groups (Ellis et al., 2008; Kekarainen et al., 2007; Kekarainen et al., 2006; Krakowka et al., 2008) is located at the both sides of the deletion. Therefore, the current nested PCR assay for PTTV2 detection is likely not sufficient to identify the genetically diverse strains of PTTV2c subtype.

#### Characteristics of the conserved amino acid motifs in the PTTV ORF1 and ORF2

The ORF1 of TTV is believed to encode a structural and replication-associated protein (Maggi and Bendinelli, 2009). The ORF1-encoding products of seven PTTV strains have 624–635 aa in length and possess a high number of arginine residues at the N-terminus that are thought to have the DNA-binding activity (Fig. 3). The predicted rolling-circle replication (RCR) motifs (Ilyina and Koonin, 1992) are presented at different positions in different PTTV types and subtypes that may be type- or subtype-specific. RCR motif-III (YxxK) is conserved in the PTTV type 1a (aa position 14–17 of PTTV1a-VA) and type 1b strains (aa position 379–382 of PTTV1b-VA), respectively (Fig. 3), whereas the same conserved motif identified in all three PTTV2 strains is located at aa position 482–485 of PTTV2b-VA (data not shown). Both PTTV2b-VA and PTTV2c-VA also have a conserved RCR motif-II (HxQ) at aa position 331–333 of PTTV2b-VA that is absent in TTV-2p (data not shown).

The ORF1 proteins of PTTV strains between species 1 and species 2 share very low aa sequence identity with only 22.4–25.8%, which makes it difficult to identify significantly conserved aa sequences between the two species. In PTTV species 1, the aa identity of ORF1 between type 1a and 1b strains are 50.3–52.7%. Sequence divergence is located throughout the ORF1 protein but relatively high number of aa substitutions were clustered in two regions at the N-terminus part (less than 30.0% and 37.5% aa similarity, respectively), in the middle part (less than 36.4% aa similarity), and at the C-terminus (less than 30.6% aa similarity) among the four PTTV1 strains (Fig. 3). No genomic regions with a relatively high number of aa substitutions were observed among the three PTTV2 strains (data not shown). The identified genomic regions in PTTV species 1 likely play a role in evading the host immune surveillance and helping PTTV to establish a persistent infection, as suggested by studies of human TTV (Nishizawa et al., 1999).

The aa sequences of ORF2 differed considerably between the four PTTV1 and three PTTV2 strains (Fig. 4). However, they share a

conserved protein-tyrosine phosphatase (PTPase)-like motif (Wx<sub>7</sub>-Hx<sub>3</sub>CxCx<sub>5</sub>H) at the N-terminus (Fig. 4) (Peters et al., 2002). This motif is also conserved among all human TTV, TTMV and TTMDV strains as well as CAV (Biagini, 2009). The TTMV or CAV ORF2 protein also exhibited a serine/threonine phosphatase (S/T PTPase) activity (Peters et al., 2002). The dual specificity of the ORF2 protein is thought to regulate host gene transcription, signal transduction and cytokine responses during viral replication. Recently, mutagenesis analyses of two conserved basic aa residues before the last histidine residue of the motif in CAV revealed that the two residues affect virus replication, cytopathology *in vitro* and attenuation *in vivo* (Peters et al., 2006; Peters et al., 2007). The two basic aa residues ("KK") are conserved in the three PTTV2 strains. However, only the first basic residue ("R") is retained in the two PTTV1a strains whereas both basic residues are substituted in the PTTV1b strains (Fig. 4). It will be very interesting to determine the role of these two basic residues in the potential pathogenesis of PTTVs with different genotypes in the future.

#### Conclusion

In summary, we have determined the full-length genomic sequences of four porcine TTV strains representing different genotypes or subtypes in a serum sample of a single boar in Virginia. The finding from this study clearly indicates that, similar to human TTV, multiple PTTV infections with distinct genotypes or subtypes exist and probably are common in pigs. We have also provided new information regarding the genomic organization, the degree of variability and the characteristics of conserved nucleotide and amino acid motifs of PTTV, which will improve the current PCR detection assay, aid in developing reagents for serological diagnostics and help initiate the structural and functional study of PTTV. A new classification of PTTV is also proposed in this study based upon the phylogenetic and genetic analyses of the genomic sequences of seven known PTTV strains.

#### Materials and methods

##### Pig sera and semen samples

Convenient serum and semen samples from 20 conventional adult boars from a Virginia pig farm were used in the study.

##### Viral DNA extraction, nested PCR and genomic PCR

Total DNA was isolated from 20 serum and 19 semen samples using QIAamp DNA mini kit (Qiagen). To screen for the positive PTTV-containing samples, nested PCR amplifications of the conserved regions in the UTR of PTTV1 and PTTV2 were initially performed by using AmpliTag Gold polymerase (Applied Biosystems) as described previously (Kekarainen et al., 2006; McKeown et al., 2004). The two primer pairs used to amplify the fragment A of PTTV1 were TTV1-mF/TTV1-mR (for the first-round PCR) and TTV1-nF/TTV1-nR (for the

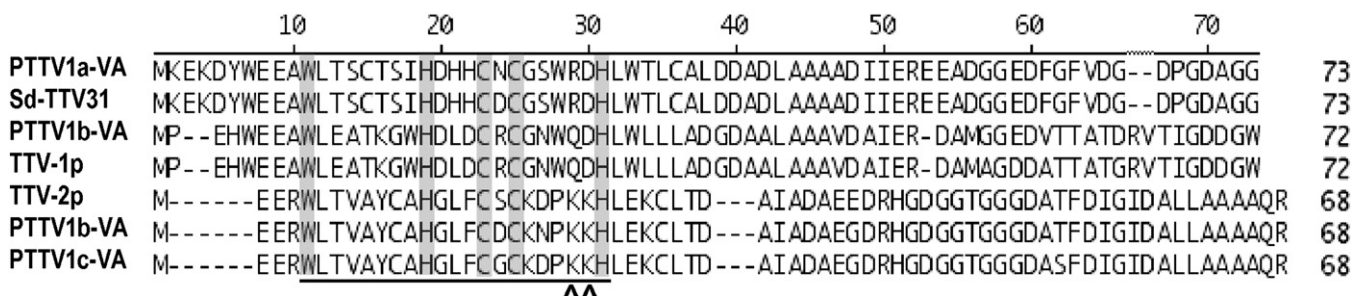


Fig. 4. Alignment of the full-length amino acid sequences of ORF2 among seven PTTV strains. Dashes indicate amino acid deletions. The five conserved amino acids within the common motif Wx<sub>7</sub>Hx<sub>3</sub>CxCx<sub>5</sub>H (underlined) identified in TTV, TTMV and CAV are shaded. The positions of the two basic aa residues before the last histidine of the motif, which have been shown to affect virus replication, cytopathology or *in vivo* attenuation in CAV, are indicated by '^'.

second-round PCR), whereas the two primer pairs used to amplify the fragment D of PTTV2 were TTV2-mF/TTV2-mR (for the first-round PCR) and TTV2-nF/TTV2-nR (for the second-round PCR; Fig. 1A and Table 1).

In order to amplify the full-length genomic sequences of both PTTV1 and PTTV2, we first performed an inverse genomic PCR using a pair of conserved gene-specific primers TTV1-IF/TTV1-IR located in region A for PTTV1 and another pair of gene-specific primers TTV2-IF/TTV2-IR located in region D for PTTV2, respectively, with Herculanase II Fusion DNA Polymerase (Stratagene) according to the manufacturer's instructions. No PCR products with expected sizes were detected. Subsequently we designed new sets of primers to amplify two regions covering the complete PTTV1 and PTTV2 genomes in the second-round PCR, respectively (Fig. 1A). The primer pairs used to amplify fragments B and C of PTTV1 were TTV1-IF/TTV1-2340R and TTV1-2311F/TTV1-TTV-IR, respectively, whereas the primer pairs used to amplify fragments E and F of PTTV2 were TTV2-IF/TTV2-2316R and TTV2-GCF/TTV2-TTV-IR, respectively (Fig. 1A and Table 1). Fragments C and F contain the GC-rich regions of PTTV1 and PTTV2, respectively. The amplified PCR products were individually excised, purified, and subsequently cloned into a pSC-B-amp/kan vector (Stratagene) by StrataClone Blunt PCR cloning strategy according to the manufacturer's instructions (Stratagene) followed by DNA sequencing.

### Sequence and phylogenetic analyses

Generic analyses and alignment of DNA and amino acid sequences were performed using Lasergene package (DNASTAR Inc., Madison, WI). The genomic sequences of three known PTTV strains and their corresponding GenBank accession numbers used for the alignment and comparison are Sd-TTV31 (AB076001), TTV-1p (AY823990) and TTV-2p (AY823991). Pairwise sequence comparisons (PASC) were performed using 121 full-length genomic sequences of human and animal TTV-related strains available in GenBank with an online program PASC (<http://www.ncbi.nlm.nih.gov/sutils/pasc/viridty.cgi?textpage=overview>) (Bao et al., 2008).

Phylogenetic trees were constructed by the neighbor-joining method in the PAUP 4.0 program (David Swofford, Smithsonian Institution, Washington, DC, distributed by Sinauer Associate Inc.) based upon the full-length genomic sequences and the deduced amino acid sequences of four ORFs of seven PTTV strains. The data were obtained from 1000 resampling.

### Acknowledgments

This study is funded by a grant from Boehringer Ingelheim Vetmedica, Inc. We wish to thank Dr. Michael Roof and Dr. Eric Vaughn of Boehringer Ingelheim Vetmedica, Inc. for their support and encouragement. We also thank Dr. M. Estienne of Virginia Tech for generously providing us with the convenient boar serum and semen samples used in the study.

### References

Bao, Y., Kapustin, Y., Tatusova, T., 2008. Virus classification by pairwise sequence comparison (PASC). In: Mahy, B.W.J., Van Regenmortel, M.H.V. (Eds.), "Encyclopedia of Virology, 5 vols.", 5. Elsevier, Oxford, pp. 342–348.

Biagini, P., 2009. Classification of TTV and related viruses (anelloriviruses). *Curr. Top. Microbiol. Immunol.* 331, 21–33.

Biagini, P., Gallian, P., Cantaloube, J.F., Attoui, H., de Micco, P., de Lamballerie, X., 2006. Distribution and genetic analysis of TTV and TTMV major phylogenetic groups in French blood donors. *J. Med. Virol.* 78 (2), 298–304.

Biagini, P., Uch, R., Belhouche, M., Attoui, H., Cantaloube, J.F., Brisbarre, N., de Micco, P., 2007. Circular genomes related to anelloriviruses identified in human and animal samples by using a combined rolling-circle amplification/sequence-independent single primer amplification approach. *J. Gen. Virol.* 88 (Pt. 10), 2696–2701.

Davidson, I., Shulman, L.M., 2008. Unraveling the puzzle of human anelloriviruses infections by comparison with avian infections with the chicken anemia virus. *Virus Res.* 137 (1), 1–15.

de Smit, M.H., Noteborn, M.H., 2009. Apoptosis-inducing proteins in chicken anemia virus and TT virus. *Curr. Top. Microbiol. Immunol.* 331, 131–149.

de Villiers, E.M., Kimmel, R., Leppik, L., Gunst, K., 2009. Intragenomic rearrangement in TT viruses: a possible role in the pathogenesis of disease. *Curr. Top. Microbiol. Immunol.* 331, 91–107.

Ellis, J.A., Allan, G., Krakowka, S., 2008. Effect of coinfection with genogroup 1 porcine torque teno virus on porcine circovirus type 2-associated postweaning multisystemic wasting syndrome in gnotobiotic pigs. *Am. J. Vet. Res.* 69 (12), 1608–1614.

Hino, S., Miyata, H., 2007. Torque teno virus (TTV): current status. *Rev. Med. Virol.* 17 (1), 45–57.

Hino, S., Prasetyo, A.A., 2009. Relationship of Torque teno virus to chicken anemia virus. *Curr. Top. Microbiol. Immunol.* 331, 117–130.

Ilyina, T.V., Koonin, E.V., 1992. Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and archaeobacteria. *Nucleic Acids Res.* 20 (13), 3279–3285.

Inami, T., Obara, T., Miyama, M., Arakawa, Y., Abe, K., 2000. Full-length nucleotide sequence of a simian TT virus isolate obtained from a chimpanzee: evidence for a new TT virus-like species. *Virology* 277 (2), 330–335.

Jelcic, I., Hotz-Wagenblatt, A., Hunziker, A., Zur Hausen, H., de Villiers, E.M., 2004. Isolation of multiple TT virus genotypes from spleen biopsy tissue from a Hodgkin's disease patient: genome reorganization and diversity in the hypervariable region. *J. Virol.* 78 (14), 7498–7507.

Kakkola, L., Hedman, K., Qiu, J., Pintel, D., Soderlund-Venermo, M., 2009. Replication of and protein synthesis by TT viruses. *Curr. Top. Microbiol. Immunol.* 331, 53–64.

Kekarainen, T., Sibila, M., Segales, J., 2006. Prevalence of swine Torque teno virus in post-weaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs in Spain. *J. Gen. Virol.* 87 (Pt. 4), 833–837.

Kekarainen, T., Lopez-Soria, S., Segales, J., 2007. Detection of swine Torque teno virus genogroups 1 and 2 in boar sera and semen. *Theriogenology* 68 (7), 966–971.

Krakowka, S., Hartunian, C., Hamberg, A., Shoup, D., Rings, M., Zhang, Y., Allan, G., Ellis, J.A., 2008. Evaluation of induction of porcine dermatitis and nephropathy syndrome in gnotobiotic pigs with negative results for porcine circovirus type 2. *Am. J. Vet. Res.* 69 (12), 1615–1622.

Leppik, L., Gunst, K., Lehtinen, M., Dillner, K., Streker, K., de Villiers, E.M., 2007. In vivo and in vitro intragenomic rearrangement of TT viruses. *J. Virol.* 81 (17), 9346–9356.

Maggi, F., Bendinelli, M., 2009. Immunobiology of the Torque teno viruses and other anelloriviruses. *Curr. Top. Microbiol. Immunol.* 331, 65–90.

McKeown, N.E., Fenaux, M., Halbur, P.G., Meng, X.J., 2004. Molecular characterization of porcine TT virus, an orphan virus, in pigs from six different countries. *Vet. Microbiol.* 104 (1–2), 113–117.

Mueller, B., Maerz, A., Doberstein, K., Finsterbusch, T., Mankertz, A., 2008. Gene expression of the human Torque teno virus isolate P/IC1. *Virology* 381 (1), 36–45.

Ng, T.F., Suedmeyer, W.K., Wheeler, E., Gulland, F., Breitbart, M., 2009. Novel anellovirus discovered from a mortality event of captive California sea lions. *J. Gen. Virol.* 90 (Pt. 5), 1256–1261.

Niel, C., Saback, F.L., Lampe, E., 2000. Coinfection with multiple TT virus strains belonging to different genotypes is a common event in healthy Brazilian adults. *J. Clin. Microbiol.* 38 (5), 1926–1930.

Niel, C., Diniz-Mendes, L., Devalle, S., 2005. Rolling-circle amplification of Torque teno virus (TTV) complete genomes from human and swine sera and identification of a novel swine TTV genogroup. *J. Gen. Virol.* 86 (Pt. 5), 1343–1347.

Ninomiya, M., Takahashi, M., Nishizawa, T., Shimosegawa, T., Okamoto, H., 2008. Development of PCR assays with nested primers specific for differential detection of three human anelloviruses and early acquisition of dual or triple infection during infancy. *J. Clin. Microbiol.* 46 (2), 507–514.

Ninomiya, M., Takahashi, M., Hoshino, Y., Ichihara, K., Simmonds, P., Okamoto, H., 2009. Analysis of the entire genomes of torque teno midi virus variants in chimpanzees: infrequent cross-species infection between humans and chimpanzees. *J. Gen. Virol.* 90 (Pt. 2), 347–358.

Nishizawa, T., Okamoto, H., Konishi, K., Yoshizawa, H., Miyakawa, Y., Mayumi, M., 1997. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. *Biochem. Biophys. Res. Commun.* 241 (1), 92–97.

Nishizawa, T., Okamoto, H., Tsuda, F., Aikawa, T., Sugai, Y., Konishi, K., Akahane, Y., Ukita, M., Tanaka, T., Miyakawa, Y., Mayumi, M., 1999. Quasispecies of TT virus (TTV) with sequence divergence in hypervariable regions of the capsid protein in chronic TTV infection. *J. Virol.* 73 (11), 9604–9608.

Okamoto, H., 2009a. History of discoveries and pathogenicity of TT viruses. *Curr. Top. Microbiol. Immunol.* 331, 1–20.

Okamoto, H., 2009b. TT viruses in animals. *Curr. Top. Microbiol. Immunol.* 331, 35–52.

Okamoto, H., Nishizawa, T., Tawara, A., Peng, Y., Takahashi, M., Kishimoto, J., Tanaka, T., Miyakawa, Y., Mayumi, M., 2000a. Species-specific TT viruses in humans and nonhuman primates and their phylogenetic relatedness. *Virology* 277 (2), 368–378.

Okamoto, H., Nishizawa, T., Tawara, A., Takahashi, M., Kishimoto, J., Sai, T., Sugai, Y., 2000b. TT virus mRNAs detected in the bone marrow cells from an infected individual. *Biochem. Biophys. Res. Commun.* 279 (2), 700–707.

Okamoto, H., Nishizawa, T., Takahashi, M., Tawara, A., Peng, Y., Kishimoto, J., Wang, Y., 2001. Genomic and evolutionary characterization of TT virus (TTV) in tupaia and comparison with species-specific TTVs in humans and non-human primates. *J. Gen. Virol.* 82 (Pt. 9), 2041–2050.

Okamoto, H., Takahashi, M., Nishizawa, T., Tawara, A., Fukai, K., Muramatsu, U., Naito, Y., Yoshikawa, A., 2002. Genomic characterization of TT viruses (TTVs) in pigs, cats and dogs and their relatedness with species-specific TTVs in primates and tupaia. *J. Gen. Virol.* 83 (Pt. 6), 1291–1297.



- Peters, M.A., Jackson, D.C., Crabb, B.S., Browning, G.F., 2002. Chicken anemia virus VP2 is a novel dual specificity protein phosphatase. *J. Biol. Chem.* 277 (42), 39566–39573.
- Peters, M.A., Crabb, B.S., Washington, E.A., Browning, G.F., 2006. Site-directed mutagenesis of the VP2 gene of Chicken anemia virus affects virus replication, cytopathology and host-cell MHC class I expression. *J. Gen. Virol.* 87 (Pt. 4), 823–831.
- Peters, M.A., Crabb, B.S., Tivendale, K.A., Browning, G.F., 2007. Attenuation of chicken anemia virus by site-directed mutagenesis of VP2. *J. Gen. Virol.* 88 (Pt. 8), 2168–2175.
- Prasetyo, A.A., Kamahora, T., Kuroishi, A., Murakami, K., Hino, S., 2009. Replication of chicken anemia virus (CAV) requires apoptin and is complemented by VP3 of human torque teno virus (TTV). *Virology* 385 (1), 85–92.
- Qiu, J., Kakkola, L., Cheng, F., Ye, C., Soderlund-Venermo, M., Hedman, K., Pintel, D.J., 2005. Human circovirus TT virus genotype 6 expresses six proteins following transfection of a full-length clone. *J. Virol.* 79 (10), 6505–6510.
- Takayama, S., Miura, T., Matsuo, S., Taki, M., Sugii, S., 1999. Prevalence and persistence of a novel DNA TT virus (TTV) infection in Japanese haemophiliacs. *Br. J. Haematol.* 104 (3), 626–629.